Commentary

BK Channel News: Full Coverage on the Calcium Bowl

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The large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) is well positioned to assume diverse physiological functions because it is ubiquitously expressed in many cell types. Some of the functions recently proposed for this channel offer unexpected surprises, however, such as a role in innate immunity in neutrophil leukocytes (Ahluwalia et al., 2004), recognition as a heme-binding protein (Tang et al., 2003), behavioral responses to ethanol (Davies et al., 2003), and a protective mechanism against ischemic cell death in the inner mitochondrial membrane of cardiac myocytes (Xu et al., 2002). These novel occupations attributed to BK_{Ca} remain to be fully explored and validated before placing them along side the well-established physiological functions of this channel in the negative feedback regulation of voltage-gated Ca^{2+} entry into nerve terminals (Robitaille et al., 1993), smooth muscle (Brayden and Nelson, 1992), and auditory hair cells of birds and turtles (Duncan and Fuchs, 2003). Nonetheless, the more we learn about the diverse roles of BK_{Ca} in the lives of cells, the greater our incentive to understand exactly how this hyperpolarizing K^+-channel protein simultaneously senses both membrane voltage and intracellular Ca^{2+}. Particularly so because BK_{Ca} is a member of a multigene family that includes Slo3, a Ca^{2+}-insensitive K^+ channel predominantly expressed in spermatocytes (Schreiber et al., 1998), and Slack and Slick, two closely related genes that encode Na^+-activated K^+ channels (Bhattacharjee et al., 2003; Yuan et al., 2003).

The surge in attention that BK_{Ca} has attracted is fueled partly by the fact that cloned BK_{Ca} genes (e.g., *Drosophila slowpoke*) are readily expressed in oocytes and cultured cell lines and that single-channel currents of BK_{Ca} can be recorded at high resolution over a wide range of open state probability. Biophysical analysis of single-channel, macroscopic, and gating currents previously led to the development of a 50-state kinetic model describing two-tiered allosteric activation of a voltage-dependent, homotetrameric channel protein that binds one Ca^{2+} ion per subunit in both closed and open states (Cox and Aldrich, 2000; Rothberg and Magleby, 2000). However, the most recent studies suggest that there are at least three Ca^{2+}-activation sites per subunit, two high-affinity, Ca^{2+}-specific sites and one low-affinity site that binds both Ca^{2+} and Mg^{2+} (Zhang et al., 2001; Bao et al., 2002; Shi et al., 2002; Xia et al., 2002). A recent review of the BK_{Ca} gating mechanism in this journal (Magleby, 2003) placed the latest tally of the "minimal" number of kinetic states at either 1,250 or 1,875, depending on whether we allow the luxury of a second tier of closed states. We are a long way afield from early modeling efforts, which attempted to limit the number of kinetic intermediates spanning the closed and open states to a meager handful (Methfessel and Boheim, 1982; Moczydlowski and Latorre, 1983). The modern kinetic analysis has provided powerful quantitative models capable of simulating the gating behavior of BK_{Ca} with high fidelity. The next challenge is the identification of the molecular basis for the different states, and several research groups have accepted this challenge by seeking biochemical evidence that may reveal structural information on the nature of the divalent cation binding sites and conformational changes that underlie the ~1,875 intermediates of BK_{Ca} gating. This issue of the *Journal of General Physiology* treats us to a fresh perspective on this endeavor in the article by Bao et al. (2004).

This article focuses on a linear sequence of 28 residues, Thr883-Gln910, near the intracellular COOH-terminal end of mSlo, the channel-forming subunit encoded by the mouse BK_{Ca} gene. The functional significance of this particular region has been a subject of intense interest since Schreiber and Salkoff (1997) first named it the "Ca^{2+} bowl" and characterized it as a highly conserved motif in BK_{Ca} that is rich in acidic residues (eight Asp and two Glu residues) and results in greatly diminished Ca^{2+}-sensitivity when altered by various mutations. Remarkably, Schreiber et al. (1999) also showed that a spliced segment containing the Ca^{2+} bowl conferred Ca^{2+} sensitivity to a chimeric channel using the normally Ca^{2+}-independent tail region of Slo3. Although the effects of various substitutions and deletions within the Ca^{2+} bowl region have been previously characterized with respect to the Ca^{2+} dependence of gating (Schreiber and Salkoff, 1997), Bao et al. (2004) now provide the first systematic alanine-scanning mutagenesis of this region. They report the results of single Ala substitutions of 20 residues within the Ca^{2+} bowl, primarily selected for the presence of oxygen-containing side chains that could potentially par-

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ticipate in the direct coordination of Ca^{2+}. The alanine scan was further performed in the background of a mutant, M513I, which was previously shown to completely suppress high-affinity responsiveness to Ca^{2+} when paired with a major deletion of the Ca^{2+} Bowl region (Bao et al., 2002). The assumption behind this latter strategic manipulation is that removal of a suspected "second site" for high-affinity Ca^{2+} activation allows the Ca^{2+}-binding potential of the Ca^{2+} bowl to be studied in virtual isolation.

Analogous to attempts to deduce the essence of voltage sensing from serial mutations of individual residues of the S4 segments of voltage-gated ion channels, these experiments offer a provocative picture of the relative importance of amino acid side chains of the Ca^{2+} bowl. If we first consider the relative reduction of the shift, $\Delta V_{0.5}$, the change in the midpoint of the voltage activation curve from 0 to 10 μM Ca^{2+} (see Fig. 4 A of Bao et al., 2004, in this issue), 10 of the bowl residues may be classified as largely extraneous "cold" positions that alter the Ca^{2+}-dependent shift of the control channel (−86 mV for M513I) by <30%. Another eight of the residues may be grouped as more interesting "medium" positions that inhibit the shift by 30–80%. Finally, two tantalizingly "hot" positions, D898 and D900, effectively abolish the shift by 95% and 84%, respectively—and these two hot residues are located in the middle of D895-P902, a "medium/hot" region that is a good candidate for a potential Ca^{2+}-binding loop (Fig. 10 of Bao et al., 2004, in this issue). These results demonstrate that certain residues of the Ca^{2+} bowl matter more than others with respect to Ca^{2+}-dependent activation and set the stage for a complementary series of follow-up experiments designed to test whether the same residues actually bind Ca^{2+}.

Unfortunately, a reliable solution-based assay for Ca^{2+} binding to soluble domains of the BK_{Ca} channel is not yet available. The only system for monitoring Ca^{2+} binding at the present time is the $^{45}Ca^{2+}$-overlay blot assay (Maruyama et al., 1984; Bian et al., 2001), a technique that has proven to reliably identify Ca^{2+}-binding proteins such as calmodulin that contain EF-hand motifs. The BK_{Ca} channel does not have any internal sequences that definitively match the requirements for a canonical EF-hand motif, although Braun and Sy (2001) have described several regions that partially fit the EF-hand stereotype. Nevertheless, the COOH-terminal 280 residues of *Drosophila* BK_{Ca} (Bian et al., 2001) and the COOH-terminal 240 residues of mouse BK_{Ca} (Braun and Sy, 2001) do exhibit a positive $^{45}Ca^{2+}$ signal in this assay that is suggestive of a bona fide Ca^{2+}-binding protein.

Surprisingly, Bao et al. (2004) found that the two hot residues, D898 and D900, with the greatest effect on $\Delta V_{0.5}$ in the electrophysiological assay of Ca^{2+} dependence, also have a large inhibitory effect on $^{45}Ca^{2+}$ binding to the COOH-terminal 240 residues of the mSlo region, 50% and 51%, respectively, as measured for single alanine mutants of these residues (see Fig. 8 A of Bao et al., 2004, in this issue). Four other "cold/medium" residues that have little effect on $\Delta V_{0.5}$, also have a small effect on the $^{45}Ca^{2+}$ signal (<30%). However, spoiling the correlation, alanine mutation of an anomalous residue, D899, that has practically no effect on $\Delta V_{0.5}$, does have a rather large inhibitory effect (55%) on the $^{45}Ca^{2+}$-binding signal. Double alanine mutations of pairs of residues, D898/D900 and D899/D901, tend to reinforce these trends. Both of these latter double mutants inhibit the $^{45}Ca^{2+}$-binding signal in the range of 73–80%.

How should these results be interpreted with respect to the hypothesis that the Ca^{2+} bowl is the physical locus of a high-affinity binding site(s) coupled to voltage activation of the channel? Perhaps it is too soon to tell. The D899A mutation, which has practically no effect on the Ca^{2+} dependence of gating but has a large effect on the $^{45}Ca^{2+}$-binding signal, is a worrisome anomaly. The authors correctly argue that the structure of the Ca^{2+} bowl may be greatly distorted in the blot assay and may not be a true reflection of Ca^{2+} binding in the native channel. This may explain certain inconsistencies between assays of Ca^{2+} activation and Ca^{2+} binding. Likewise, mutated residues that affect the Ca^{2+} dependence of voltage gating need not be located directly within a Ca^{2+}-binding site to perturb this process. However, when taken together with the demonstration that chimeric insertion of a region containing the Ca^{2+} bowl confers Ca^{2+} sensitivity to the Slo3 Tail (Schreiber et al., 1999), the probability that all of these results are due to a coincidental allosteric effect on Ca^{2+} binding to a region that lies outside of the Ca^{2+} bowl seems rather remote.

What kind of evidence is required to finally prove the Ca^{2+} bowl hypothesis and unravel the molecular mechanism of Ca^{2+} activation of BK_{Ca}? In the short term, one line of experiments that may be helpful would be to compare the $^{45}Ca^{2+}$-binding signal of the whole COOH-terminal cytoplasmic domain of BK_{Ca} to that of various partial fragments of this same region using the protein blot technique. Some of these partial fragments should be constructed to contain the separate and combined RCK1 and RCK2 regions homologous to the distantly related MthK K^{+} channel (Jiang et al., 2002). Such studies might identify particular regions of the intracellular domain of the BK_{Ca} protein that exhibit the highest levels of $^{45}Ca^{2+}$-binding activity and thus reveal the approximate location of the various Ca^{2+}-binding sites. However, this approach assumes that a gel-blot assay using SDS-denatured protein is capable of identifying Ca^{2+}-binding sites that exist in the native protein.

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In the longer term, it will be necessary to determine the three-dimensional structure of the BK_{Ca} channel or parts thereof, i.e., the whole intracellular domain, and/or subdomains of the intracellular domain that bind Ca^{2+}. An ultimate goal of biochemical analysis of the BK_{Ca} channel would be to replace the $^{45}Ca^{2+}$-blot assay with a solution-based assay for Ca^{2+} binding, such as equilibrium dialysis or a spectroscopic method. These are formidable tasks, given the difficulty of expressing large fragments of the BK_{Ca} channel in soluble form. However, all of the current work on the Ca^{2+} bowl may be virtually irrelevant if the findings of Piskorowski and Aldrich (2002) are confirmed. These latter workers reported that the normal Ca^{2+} and voltage dependence of gating are practically unaltered when the whole cytoplasmic COOH-terminal region is deleted from the channel protein, implying that the Ca^{2+}-binding sites are actually located within the S0–S6 membrane domain. To settle this controversy, a tenacious scientific team needs to gear up and charge into the Ca^{2+} bowl, ignore the distractions of the half-time show, and prevail over the technical impediments that hinder progress on this important problem.

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